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First molecular evidence of bovine ephemeral fever virus in Sindh, Pakistan: phylogenetic insights and risk factor correlation

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Abstract

Bovine ephemeral fever (BEF) is a re-emerging arbovirus infection, causing substantial economic losses primarily attributed to decreased milk production. BEF is a frequently reported disease in Pakistan, however, there is a lack of scientific data, particularly regarding gene sequencing and phylogenetic analysis of this viral infection in bovines. This research was planned for the molecular detection of the BEF virus in dairy animals of Sindh province, Pakistan. The association of various risk factors, including seasonal variation, species, breed, sex, age, lactation and feeding pattern was also analyzed. A total of 470 blood samples from cattle and buffaloes were collected across different topographical regions of Sindh from 2021-2022; and subjected to molecular detection through RT-PCR. Sequencing of positive RT-PCR amplicons and phylogenetic analysis were then performed. A questionnaire was designed to get comprehensive information about each animal before sample collection to develop an association between various risk factors. RT-PCR amplification of the *glycoprotein* gene yielded a 420 bp product and the phylogenetic tree revealed that the Pakistani BEF virus isolates clustered closely with strains from Iran, India and Australia indicating a strong genetic resemblance. The overall prevalence was 13.83% (65/470) which significantly higher in cattle (15.49%) than in buffaloes (3.70%), and cattle had significantly higher chance of infection than buffaloes (odds ratio = 4.31, confidence interval = 1.33 - 13.95). This study provided the first comprehensive gene sequencing data on BEF in Pakistan, significantly enhancing the global understanding of the virus through detailed molecular and phylogenetic analysis.

Introduction

Livestock has emerged as the largest subsector of agriculture in Pakistan, providing jobs to more than 8.00 million people. The livestock sector holds a significant share in Pakistan economy.[1] Cattle and buffalo are the two major shareholders of the dairy industry, with approximately 57.50 and 46.30 million populations, respectively, and their share in total milk production is about 67.98 million tons.[2]

The livestock sector in Pakistan is facing numerous challenges, including disease outbreaks, managemental issues and adverse climatic conditions, all of which hinder the growth and productivity of the sector. One of the biggest challenges is the prevalence of infectious diseases and lack of proper diagnostics and control measures. Bovine ephemeral fever (BEF) is one of the major re-emerging diseases in Pakistan, severely affecting animal productivity and profitability. It is an insect-borne, non-contagious, and dreadful viral disease mainly affecting cattle and water buffalo. This disease is known as a three-day sickness due to its very short course of duration.[3]

Three-day sickness is caused by BEF virus (BEFV), a bullet-shaped, negative-sense, single-stranded RNA virus that belongs to the genus *Ephemerovirus* within the *Rhabdoviridae* family.[4] The BEFV has five to six genes encoding for five structural proteins named as glycoprotein (G), L-protein, which is RNA-dependent RNA polymerase, nucleocapsid protein, polymerase-associated protein, and viral matrix protein.[5] Molecular detection can be made by amplifying the *G* gene using a traditional RT-PCR.[6]

The BEF exhibits a widespread distribution, encompassing extensive regions across Australia, Middle East, Africa, and Asia. However, the disease tends to be more concentrated and endemic within the tropical,

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subtropical, and temperate regions of these continents.[7] Pakistan, being in a subtropical region and its susceptibility to floods exacerbate the risk of disease outbreaks like BEF.[8] Various species of mosquitoes and biting midges are the most important vectors for transmission of BEFV.[6] Epidemiological investigations are strongly suggestive of the seasonal occurrence of the disease with increased prevalence in wet and rainy seasons, especially during heavy monsoons.[9] The monsoon rains of 2022, coupled with flooding were record-breaking in Pakistan history. These floods resulted in significant damage to the livestock industry, particularly through disease outbreaks.[8]

The BEFV mainly affects cattle (*Bos* species) and water buffalo (*Bubalus bubalis*), but cattle are considered to be more vulnerable to infection as compared to buffalo.[3] Sex, age, breed, physiological status (lactation and pregnancy status), and seasonal variation, along with the proliferation of vector populations are reported as the most significant risk factors for this infection.[10,11] The direct economic losses in terms of mortality are very low, however, considerable losses occur due to decline in milk production, loss of fertility in males, abortion in case of pregnant animals, loss of draught power, cost of treatment, and implications of trade restrictions.[12] This acute febrile condition is characterized by the sudden onset of fever, a significant drop in milk yield, nasal discharge, lameness and sometimes prolonged recumbency. Affected animals generally show signs of depression, anorexia, profuse salivation, shifting lameness, muscular tremors and enlargement of lymph nodes.[7] The mortality rate is generally low (1.00 - 2.00%), but the morbidity rate is very high, which may go up to 80.00 - 100%.[11]

Field diagnosis of BEF is based on the history of sudden outbreaks in a herd and clinical manifestations of a disease, as this infection is mainly associated with seasonal variation, where the vector proliferation is very high.[13] Serological diagnosis can be made by detecting rising titers of neutralizing antibodies in paired serum samples.[6] BEF was first reported in Pakistan in 1999 in Faisalabad region of Punjab province.[14] Subsequently, sporadic cases of the disease continued to emerge due to the lack of awareness among farmers and poor diagnostic facilities, which eventually caused the livestock industry to suffer significant economic losses. Only two studies have been reported from Punjab province and one from Khyber Pakhtunkhwa, Pakistan.[10,15] The comprehensive research efforts remained limited in other provinces of Pakistan. Sindh province has remained neglected in the perspective of disease surveillance and control programs.[16] It is located in a tropical region with subtropical areas in the north, and experiences hot, humid summers with substantial rainfall and cold, dry winters. Temperatures often surpass 46.00 ˚C from May to August, dropping to an average low of 2.00 ˚C in December and January. Annual rainfall averages nearly 14.00 inches (360 mm), clustered mainly between June and September. Moreover, the climatic conditions of Sindh province, coupled with its geographical location, render it susceptible to heavy monsoons and floods. These conditions create a conducive environment for insect vectors, increasing the likelihood of outbreaks of various diseases.[8] This study was designed for molecular detection of BEFV in Sindh province, Pakistan, its phylogenetic analysis and to determine association between BEF and various risk factors contributing to the occurrence of this disease.

Materials and Methods

Study area. For this study, four districts (Sukkur, Khairpur, Sanghar, and Larkana) were specifically chosen. Samples were collected from dairy animals present in the above-mentioned regions of Sindh, Pakistan.

Data collection. A questionnaire was designed to get comprehensive information about each animal before sample collection. This questionnaire was comprised of multiple sections, including signalment, physiological details, husbandry and climatic environmental data. Signalment data included basic information like species (Cattle and buffalo), sex (male, female), breed (Crossbred, Red Sindhi, Tharparkar, and Kundi) and age (< 1, 1 - 2, 3 - 4 and > 4 years). Physiological data included information regarding body weight (50.00 – 150, 151 - 250, 251 - 350, 351 - 450 and > 451 kg), gestation (pregnant, non-pregnant), production status (dry, milking) and number of lactation (1^{st}, 2^{nd}, 3^{rd}, and > 4^{th}) to establish a relationship of disease with the physiological status of animals. Information regarding feeding patterns (grazing, stall-fed, combined) was recorded to check the effect of management factors on disease occurrence. In climatic and environmental factors seasonal variation, especially rainfall, was recorded to check their effect on disease occurrence in selected regions.

Sample collection. A total of 470 animals were randomly selected using convenient sampling, regardless of disease status, considering the short clinical course and viral persistence. Out of 470, 389 samples were collected from cattle and 81 from buffaloes. Serum samples were collected from both healthy and diseased animals, transported by maintaining a cold chain and stored at – 20.00 ˚C until further analysis in the Veterinary Preventive Medicine and Public Health Laboratory, University of Agriculture, Faisalabad. Formal approval was sought from the Institutional Bioethics Committee, University of Agriculture, Faisalabad, for this study as *per* letter number 7924/ORIC.

Molecular detection RT-PCR of BEFV *G*-gene. Molecular diagnosis and confirmation of BEF were done from serum samples using RT-PCR in the Veterinary Preventive Medicine and Public Health Laboratory,

University of Agriculture Faisalabad. RNA was extracted using a viral nucleic acid extraction kit (Alphagen Biotech, Changzhi, Taiwan) according to the manufacturer's description. Extracted RNA was amplified using Prime-Script™ One-Step RT-PCR kit (Takara Biomedical Technology, Beijing, China). The amplification of the *G* gene was done by using forward and reverse primers as previously described.[17] Primers (420F5´AGAGCTTGGTGTG AATAC3´ and 420R 5´CCAACCTACAACAGCAGATA3´were used for one-step RT-PCR with the following conditions: initial incubation at 50.00 ˚C for 30 min, initial denaturation 94.00 ˚C for 2 min and 35 cycles of denaturation at 94.00 ˚C for 30 sec, annealing at 55.00 ˚C for 30 sec, extension at 72.00 ˚C for 1 min and a final extension at 72.00 ˚C for 5 min. The amplified products were analyzed through agarose gel electrophoresis. DNA samples were extracted from the gel using Thermo Scientific™ GeneJET Gel Extraction Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) following the manufacturer's guidelines.[18]

Sequencing and phylogenetic analysis. Sequencing of positive PCR amplicons was carried out using the Celemics Korean commercial service. The sequences were analyzed using the PubMed tool with the same 420F primer in both directions and the obtained sequences were submitted to the NCBI GenBank database for accession numbers. The BEFV sequences isolated from Pakistan during this study and reference sequences from other countries obtained from the NCBI Genebank database were aligned using Clustal W Multiple Sequence Alignment Program (UCD, Dublin, Ireland).[19] The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model.[20] Bootstrap values (%) showed at the branches represent the proportion of replicate trees in which the associated taxa clustered together. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model and then selecting the topology with superior log likelihood value. This analysis involved 17 nucleotide sequences. There was a total of 14,935 positions in the final dataset. Evolutionary analyses were conducted in MEGA Software (version 11.0; Biodesign Institute, Tempe, USA).[21]

Statistical analysis. The chi-square test was applied to check the significant effect of various risk factors associated with disease prevalence. The upper and lower limits of the 95.00% confidence interval for prevalence percentage were measured with WinPepi Software (version 11.65; Brixton Health, London, UK) was used to compute the odds ratio to measure the relative risk of disease in the study population. The logistic regression model was applied using SAS Software (version 9.4; SAS Institute, Cary, USA) to check the significance of various risk factors associated with disease prevalence. Moreover, logistic analysis was also applied through backward and forward elimination methods to draw inferences about diverse risk factors with BEF in cattle and buffaloes.

Results

RT-PCR and phylogenetic analysis. The identification of BEFV was done through RT-PCR using specific primers targeting the *G* gene and resulted in a single distinct band of cDNA amplification. These products were visualized under ultraviolet light as shown in Figures 1 and 2. A total of 65 (13.83% of 470 sampled) were detected as positive on RT-PCR amplification. Among these 65 samples, 60 (92.30%) were clinically infected and 5 (7.70%) were clinically normal at the time of sampling. The amplified product size was 420 bp, corresponding to the entire gene length. The BEFV isolates obtained during this study from Pakistan were represented by accession numbers OR854821, OR854822, OR854823, and OR854824. The phylogenetic tree was constructed indicating that all four Pakistani isolates closely resembled those from Iran, India, and Australia. Moreover, these isolates also showed some genetic variation among themselves (Fig. 3).

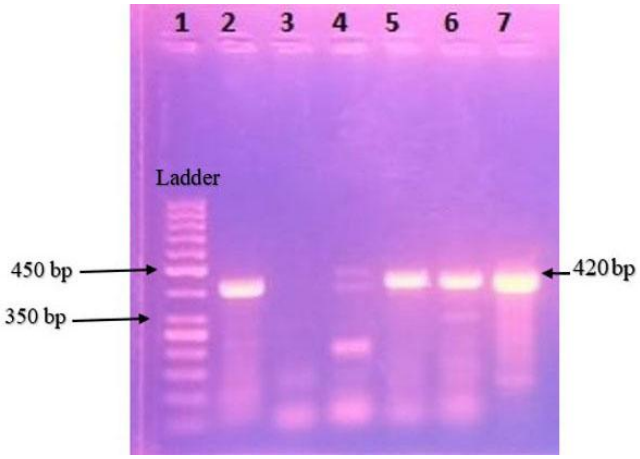

Fig. 1. Agarose gel electrophoresis image of RT-PCR amplicons of *G* gene of BEFV isolates from Pakistan, Lane 1: Marker 50 bp; Lane 2: Control positive; Lane 3: Control negative; Lane 4: Negative sample; Lanes 5-7: BEFV amplicons of 420 bp.

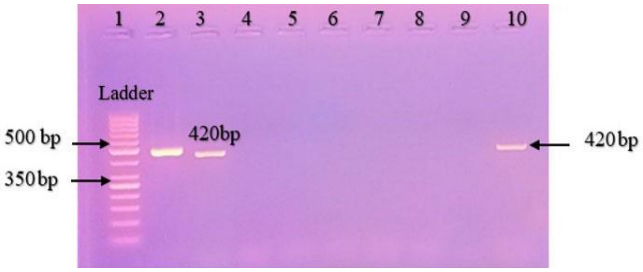

Fig. 2. Agarose gel electrophoresis RT-PCR products of *G* gene of BEFV isolates from Pakistan, Lane 1: Marker 50 bp; Lane 2: Control positive; Lanes 3 and 10: BEFV amplicons of 420 bp; Lane 4-9: Negative samples.

Genetic similarities with neighboring countries. Blast analysis of the isolates obtained in this study revealed that out of four, the first three sequences shared approximately 99.00% homology with Iranian (OR509494) and Indian (MN839987) isolates, showing a nucleotide substitution at 395, where adenine was replaced by guanine. Similarly, the fourth sequence (OR854824) showed 99.00% homology with Australian strain (MN026882) with nucleotide differences observed at three positions.

Prevalence of BEF and associated risk factors. Cattle and buffalo were the most affected species by BEF and the overall prevalence was 13.83% (65/470). Species-specific prevalence was significantly higher in cattle (15.49%) than in buffaloes (3.70%). The analysis of all variables at the individual level indicated that sex, breed, age, body weight, physiological status, season and feeding were significant risk factors showing a close association with disease prevalence in cattle, while non-significant in buffaloes (Tables 1 and 2). The highest disease prevalence was recorded in the 3 to 4 years age group, body weight 351 - 450 kg, pregnant, lactating animals fed on grazing in September. The non-significant variable (area) was eliminated according to the fit model, whereas, the significant variables for disease occurrence in cattle species are represented in Table 3. Multivariate logistic regression analysis revealed that breed, sex, body weight, production status, type of feeding and season were significantly associated with disease occurrence, revealing that crossbred and female animals showed high disease susceptibility compared to indigenous and male animals. Similarly, older and heavier animals and those in active production status were found to be more frequently affected. Feeding pattern revealed that animals managed under free-grazing systems showed significantly higher infection rate during and post monsoon season.

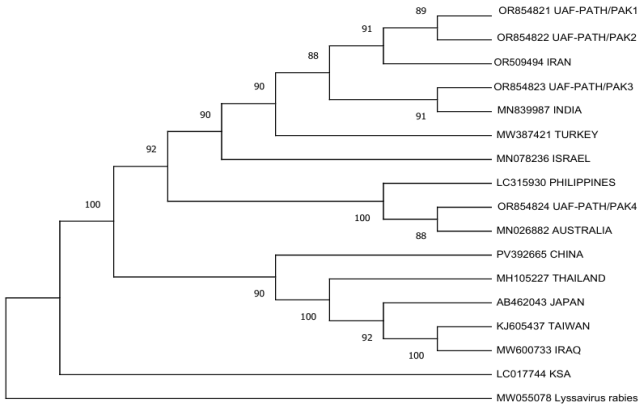

Fig. 3. Phylogenetic tree of bovine ephemeral fever virus based on partial sequencing of *G* gene of four Pakistani isolates.

Table 1. Univariate analysis of different risk factors for the molecular prevalence of bovine ephemeral fever in cattle.

Variables	Categories	RT-PCR positive	Total	Prevalence % (95.00% CI)	Odds Ratio (95.00% CI)	χ^2 (*p*-value)
Cattle	Female	58	311	18.65 (14.71 - 23.35)	3.64 (1.29 - 10.26)	6.65 (0.01)
	Male	4	78	5.13 (2.01 - 12.46)	Ref	
Breed	Crossbred	37	167	22.16 (16.53 -29.05)	2.47 (1.06 - 5.76)	6.44 (0.03)
	Red Sindhi	18	144	12.50 (8.06 - 18.89)	1.39 (0.56 - 3.46)	
	Tharparkar	7	78	8.97 (4.41 - 17.78)	Ref	
Age	3 - 4 Years	33	145	22.76 (16.69 - 30.24)	31.08 (1.92 -504.44)	14.29 (0.01)
	1 - 2 Years	21	122	17.21 (11.54 -24.88)	23.69 (1.44 -389.26)	
	> 4 Years	8	55	14.55 (7.56 - 26.17)	20.68 (1.19 -358.47)	
	< 1 Year	0	67	0.00 (0.00 - 5.42)	Ref	
Body weight	351 - 450	24	94	25.53 (1.78 - 35.18)	34.48 (2.10 -565.11)	17.97 (0.01)
	> 450	5	23	21.74 (9.66 - 41.90)	31.13 (1.70 -570.35)	
	251 - 350	23	112	20.54 (1.41 - 28.94)	27.78 (1.70 -455.35)	
	151 - 250	10	94	10.64 (5.88 - 18.49)	14.78 (0.87 -251.25)	
	50 - 150	0	66	0.00 (0.00 - 5.50)	Ref	
Physiological status	Pregnant	33	159	20.75 (15.17 - 27.71)	1.26 (0.72 - 2.22)	1.10 (0.29)
	Non-pregnant	25	152	16.45 (11.40 - 23.16)	Ref	
Pregnant	Milking	20	79	25.32 (17.03 - 35.90)	1.45 (0.69 - 3.05)	0.94 (0.33)
	Dry	14	80	17.50 (10.72 - 27.26)	Ref	
Non-pregnant	Milking	17	51	33.33 (21.97 - 47.03)	4.81 (1.88 - 12.28)	12.15 (0.01)
	Dry	7	101	6.93 (3.40 - 13.62)	Ref	
Lactation	3rd	10	25	40 (23.40 - 59.26)	11.12 (0.67 -184.81)	6.05 (0.10)
	2nd	15	60	25 (15.78 - 37.23)	6.92 (0.43 -111.04)	
	1st	13	72	18.06 (10.87 -28.49)	5.03 (0.31 - 81.09)	
	4th	0	13	0.00 (0.00 - 22.81)	Ref	
Type of feeding	Grazing	37	155	23.87 (17.84 - 31.16)	4.54 (1.57 - 13.11)	10.72 (0.01)
	Stall-fed	21	158	13.29 (8.86 - 19.46)	2.53 (0.84 - 7.57)	
	Combined	4	76	5.26 (2.06 - 12.76)	Ref	

Table 1. Continued.

Variables		Categories	RT-PCR positive	Total	Prevalence % (95.00% CI)	Odds Ratio (95.00% CI)	χ^2 (*p*-value)
Area		Larkana	17	65	26.15 (17.02 - 35.95)	2.84 (1.12 - 7.23)	7.77 (0.05)
		Sukkur	28	150	18.67 (13.25 -25.66)	2.03 (0.85 - 4.83)	
		Khairpur	10	98	10.20 (5.63 -17.77)	1.11 (0.41 - 3.03)	
		Sanghar	7	76	9.21 (4.53 - 17.81)	Ref	
Seasonal variation	**Larkana**	September	9	15	60 (35.75 - 80.18)	4.80 (1.15 - 20.04)	6.03 (0.04)
		August	5	26	19.23 (8.51 - 37.88)	1.54 (0.34 - 6.95)	
		July	3	24	12.5 (4.34 - 31.00)	Ref	
	Sukkur	September	15	45	33.33 (21.36 - 47.93)	3.33 (1.03 - 10.74)	6.06 (0.04)
		August	9	65	13.85 (7.46 - 24.27)	1.38 (0.40 - 4.73)	
		July	4	40	10.00 (3.96 - 23.05)	Ref	
	Larkana	September	5	20	25.00 (11.19 - 46.87)	10.75 (1.21 - 95.34)	6.04 (0.04)
		August	4	35	11.43 (4.54 - 25.95)	4.91 (0.54 - 44.80)	
		July	1	43	2.33 (0.41 - 12.07)	Ref	
	Sanghar	September	4	14	28.57 (11.72 -54.65)	10.00 (1.07 - 93.72)	6.01 (0.04)
		August	2	27	7.41 (2.06 - 23.37)	2.59 (0.23 - 29.02)	
		July	1	35	2.86 (0.51 - 14.54)	Ref	

p value > 0.05 = Non significant; CI: Confidence interval.

Table 2. Univariate analysis of different risk factors for the molecular prevalence of bovine ephemeral fever in buffaloes.

Variables		Categories	RT-PCR positive	Total	Prevalence % (95.00% CI)	Odds Ratio (95.00% CI)	χ^2 (*p*-value)
Sex		Male	1	11	9.09 (1.62 - 37.73)	3.18 (0.29 - 35.29)	0.92 (0.33)
		Female	2	70	2.86 (0.79 - 9.84)	Ref	
Breed		Kundi	3	81	3.70 (12.70 - 10.33)	-	-
Age		3 - 4 Years	2	28	7.14 (1.98 - 22.64)	1.67 (0.08 - 32.90)	1.92 (0.58)
		1 - 2 Years	1	24	4.17 (0.74 - 20.25)	1.16 (0.05 - 27.01)	
		> 4 Years	0	20	0.00 (0.00 - 16.11)	-	
		< 1 Year	0	9	0.00 (0.00 – 29.91)	Ref	
Body weight (kg)		251 - 350	1	8	12.50 (2.24 - 47.09)	8.29 (0.34 - 203.19)	4.06 (0.39)
		351 - 450	1	9	11.11 (1.99 - 4.35)	7.42 (0.30 - 181.35)	
		> 450	1	27	3.70 (0.66 - 18.28)	2.56 (0.11 - 61.79)	
		151 - 250	0	14	0.00 (0.00 - 21.53)	-	
		50 - 150	0	23	0.00 (0.00 - 14.31)	Ref	
Production status		Pregnant	2	26	7.69 (2.13 -24.14)	8.40 (0.40 - 174.91)	3.23 (0.07)
		Non-Pregnant	0	44	0.00 (0.00 - 8.03)	Ref	
		Milking	2	11	18.18 (5.14 - 47.70)	6.74 (0.33 - 139.28)	2.49(0.11)
		Dry	0	15	0.00 (0.00 - 20.39)	Ref	
Lactation status		2nd	1	9	11.11 (1.99 - 4.35)	1.22 (0.08 - 19.54)	0.02 (0.89)
		1st	1	11	9.09 (1.62 - 37.73)	Ref	
Type of feeding		Grazing	2	32	6.25 (1.73 - 20.15)	3.77 (0.18 - 77.41)	1.42 (0.49)
		Stall-fed	1	25	4.00(0.71 - 19.54)	2.88 (0.12 - 69.58)	
		Combined	0	24	0.00 (0.00 - 13.80)	Ref	
Area		Khairpur	1	17	5.88 (1.05 - 26.98)	1.97 (0.08 - 46.45)	0.78 (0.85)
		Larkana	1	19	5.26 (0.93 - 24.63)	1.77 (0.08 - 41.64)	
		Sukkur	1	34	2.94 (0.52 - 14.91)	1 (0.04 - 23.43)	
		Sanghar	0	11	0.00 (0.00 - 25.88)	Ref	
Seasonal variation	**Khairpur**	September	1	9	11.11 (1.62 - 37.73)	1.42 (0.06 - 29.48)	0.85 (0.65)
		August	0	4	0.00 (0.00 - 48.99)	Ref	
		July	0	4	0.00 (0.00 - 48.99)	-	
	Larkana	September	1	12	8.33 (1.49 - 35.38)	1.32 (0.06 - 29.48)	0.57 (0.75)
		August	0	5	0.00 (0.00 - 43.45)	Ref	
		July	0	2	0.00 (0.00 - 65.76)	-	
	Sukkur	September	1	21	4.76 (0.85 - 22.67)	1.19 (0.05 - 27.37)	0.61 (0.73)
		August	0	8	0.00 (0.00 - 32.44)	Ref	
		July	0	5	0.00 (0.00 - 43.45)	-	

p value > 0.05 = Non significant; CI: Confidence interval.

Table 3. Logistic regression analysis for risk factors having significant association.

Variables	Odds ratio	95.00% confidence limits (Lower - Upper)	*p*-value
Logistic regression (Cattle)			
Breed	3.67	1.80 - 7.48	0.0003
Age	0.20	0.10 - 0.41	< 0.0001
Bodyweight (kg)	1.02	1.01 - 1.03	< 0.0001
Production status	3.31	1.21 - 9.09	0.02
Type of feeding	0.32	0.16 - 0.67	0.0021
Season	3.17	1.87 - 5.39	< 0.0001
Multivariate logistic regression including age and individual variables in the model			
Sex	3.47	1.19 - 10.17	0.02
Breed	1.45	1.06 - 2.01	0.02
Bodyweight (kg)	1.01	1.01 - 1.02	0.0001
Production status	4.06	1.87 - 8.87	0.0004
Type of feeding	0.44	0.29 - 0.68	0.0002
Season	2.89	1.94 - 4.31	< 0.0001

Discussion

This study provided the first molecular confirmation and phylogenetic characterization of BEFV in Pakistan, filling a major gap in the literature. BEFV was detected among large ruminants by employing RT-PCR in the Philippines and phylogenetic analysis showed 99.00% homology with the Australian strain.[22,23] Previous regional reports from neighboring countries such Iran,[5] and Philippines,[22] and as India,[24] have confirmed the presence of BEFV using molecular tools. However, Pakistan currently lacks published molecular data, particularly in terms of BEFV sequencing. By partial sequencing of *G* gene and submitting four partial sequences (OR854821, OR854822, OR854823, OR854824), we offered foundational genetic data for future epidemiological studies in Pakistan. The phylogenetic analysis revealed that the Pakistani isolates were clustered closely with Middle Eastern and Australian lineages, particularly strains from Iran,[5] Australia,[6] and India.[24] Moreover, these four sequences also had slight variations among themselves. The results of this study indicated that the same Australian or Philippian virus might be responsible for disease occurrence in Sindh, as many animals are imported from these countries. BEF was first reported from India in 2021 and showed close resemblance with isolates from Middle Eastern countries, including Iran, Turkey, the Kingdom of Saudi Arabia (KSA), and Israel.[24] In the same disease, BEFV isolated from Iran in 2021 showed homology with India, Turkey and Israel in the Middle East cluster.[5] BEF strain caused massive disease outbreaks in KSA and also showed a close relation with the Australian strain.[25] It could be concluded that the same BEFV circulating in the Middle East region is responsible for disease outbreaks in these countries sharing common borders. Moreover, the genomic fragments obtained during this study were limited in number and small in size suggesting that future studies are required to get larger genome sequences at the national level for a better understanding of BEFV in Pakistan.

Despite Sindh climate and ecology being highly conducive to BEF outbreaks, characterized by high humidity, monsoon flooding and vector-dense irrigation systems, prior Pakistani studies have not focused on these risks. The present study addressed that gap by providing first comprehensive regional assessment of BEF prevalence and risk factors in flood-prone areas. Cattle were more susceptible to contracting BEF compared to buffaloes across all studied regions and these findings were consistent with previous reports showing higher infection rate in cattle.[23,25] The same trend of higher infection rates in cattle than in buffalo was reported previously.[17] It was observed that cattle exhibited more frequent and more severe clinical signs than buffaloes in various dairy farms in Egypt from July to August.[26] The possible reason for this difference in susceptibility might be due to the difference in skin characteristics of both species. Buffalo skin is three times thicker compared to cattle indicating its strong and durable texture and therefore it is less affected by mosquito bites which is an important factor for disease transmission.[27,28]

The BEF prevalence was found to be significantly higher in female cattle (18.65%) than in males (5.13%), while in the case of buffalo, an opposite trend was recorded, with higher prevalence in males (9.09%) than females (2.86%). Similarly, previous reports from Egypt also showed higher BEF incidence in female animals compared to male animals.[29] The BEF infection was reported to be the same (49.11%) in both sexes in Punjab, Pakistan.[10] The prevalence of BEF was recorded in cows and buffaloes in Iran, suggesting that both sexes can get infected, but the infection rate was found to be higher in females.[28] However, higher sero-prevalence was reported in male cattle as compared to females in KSA and Turkey.[13,30] The major reason for more disease prevalence in female animals is the difference in their physiological status, as female animals are in various stressful conditions like pregnancy, lactation, etc., which make them more prone to infections.[26]

Crossbred cattle exhibited a significantly higher prevalence rate (22.16%) compared to indigenous Tharparkar (8.97%) and Red Sindhi (12.50%) breeds and only one local breed of buffalo was prevalent in target districts having a prevalence rate of 3.70%. A higher disease prevalence was documented among Friesian cows (13.46%) compared to native breeds (6.41%) of cows in Egypt.[29] These findings were consistent with the previous study,[25] which recorded a higher frequency of BEF infection in exotic breeds compared to native breeds. Exotic breeds are more prone to BEF infection compared to local breeds of cattle during an outbreak in India.[31] The major difference in their prevalence is mainly due to hereditary characteristics of breeds. Indigenous breeds have adapted to the local environment, while crossbred cattle exhibited higher disease susceptibility due to their low resistance against diseases and struggle with Sindh extreme temperature, frequently exceedingly more than 40.00 ˚C pre-monsoon, and smallholders lack resources for adequate cooling systems.[22] The skin of buffalo is thicker than cattle, reducing vector bites, a critical factor in Sindh flood-prone areas, where vector density peaks after monsoon. Moreover, wallowing behavior of buffaloes is also a major reason for low prevalence in the studied areas.[28]

The present study revealed an age-related variation in BEF infection, as higher infection was recorded in animals aged 3 - 4 years, and no infection was observed in animals less than one year of age in both cows and buffaloes. The results of the current study are supported by various studies from Saudi Arabia,[32] and Israel,[33] which have shown a higher disease incidence across adult animals compared to younger animals. Old age was described as a significant risk factor affecting the prevalence of BEF in previous reports.[4,34] Similar trends were recorded in Jordan, where seropositivity of BEF was higher in animals aged 1 - 4 years (36.00%) than in animals over 4 years (14.00%),[5] and in Egypt age group above 2 - 4 years was reported as a highly significant risk factor for BEF.[29] The lower disease prevalence among young animals might be due to the presence of maternal antibodies, making them more naturally resistant to infection.[34] It was documented that the younger calves were naturally resistant to BEF infection.[33] The calves of age group five months or below had shown protection against BEFV infection due to the presence of high levels of maternal antibodies from previously naturally infected or vaccinated mothers. The weakened immune response of old animals makes them more susceptible to diseases.[32]

It was seen that lactating animals were more susceptible to infection as compared to heifers and dry or non-lactating animals. Moreover, BEF infection was recorded more among pregnant cattle and buffaloes as compared to non-pregnant animals. These results were consistent with previous reports,[34] where BEF incidence was higher among lactating females in Iraq. Dairy animals in peak lactation were found to be infected with BEF following an outbreak in El-Dalangat, Behera Governorate. BEFV infection was reported in pregnant as well as lactating cows during previous studies, indicating production as a major stress factor for disease occurnace.[35] The increased susceptibility to infection among pregnant and lactating animals, and the increased physiological and biological stress and hormone fluctuations could be an important contributing factor to more disease occurrence among this group.[26] It is a common feature of viral infections that an etiological agent is more likely to cause diseases due to various stress factors that are associated with early lactation and calving process.[36,37]

Feeding patterns also influence disease risk as BEF was more prevalent among grazing cattle, followed by stall-fed and combined patterns (stall-fed + grazing) of feeding. The animals that go for grazing in open areas are more vulnerable to mosquito bites which are an important vector for disease.[38,39] Studies have suggested that mosquitoes are the most likely biological vectors of the virus and their population is high in areas that have water aggregation.[38]

The current investigations indicated that the highest prevalence of BEF was noted in September, followed by August and July among the cattle. Buffaloes experienced the highest BEF infection in September. The BEF outbreaks were observed in early autumn and followed by heavy summer rainfalls in Sindh. Larkana experienced the highest rainfall of 762 mm, while 698-, 606-, and 716-mm rainfall were recorded in Sukkur, Khairpur, and Sanghar, respectively. These districts of Sindh experienced heavy monsoon rainfall leading to the great flood of 2022.

The present findings were similar to the previous reports,[4] which showed that BEF outbreaks were reported more frequently after monsoon rains. BEF outbreaks were recorded in various cattle farms in Madhya Pradesh, India, from July to September following monsoon rains.[31] A similar trend of BEF infection was seen following heavy rainfall in three districts of Punjab, Pakistan, in August 2019, indicating Pakistan and India share the same seasonal variations, which is a vital component for the prevalence of the disease in these regions.[27]

Cattle and buffalo were more likely to suffer from BEF infection by the end of summer following the monsoon season in various districts of Egypt.[18] The BEF infection was seen in August and September, receiving high precipitation rates in various regions of Israel.[33] A high infection rate was witnessed by the end of the summer months in subtropical regions of KSA.[30] In the same way, an elevated frequency of BEF was noted during months experiencing heavy rains.[40,41]

The BEF outbreaks were seen in late autumn, followed by heavy rainfall in summer. The studied districts are located in low-lying areas that are prone to floods and

experienced heavy rain in 2022. The heavy rainfall of 2022 led to devastating floods in Sindh, creating a favorable environment for the proliferation of disease vectors like mosquitoes and midges. Moreover, the stagnant water after heavy storms resulted in an increased population of mosquito vectors, which contributed to the transmission and spread of BEFV among cattle and buffalo populations. A humidity level of 75.00% and a temperature of 25.00 - 30.00 ˚C are ideal requirements for mosquitoes' growth and active proliferation.[42,43] These findings supported the result of our study that moderate temperature, high humidity and flooded areas, all factors collectively increased the risk of proliferation and breeding places of insect vectors.

The BEF epidemics reported in neighboring countries such as Afghanistan, Iran, China and India likely facilitated the introduction of the virus into the studied regions of Pakistan.[31,40,41] There is evidence for intercontinental transmission of insect vectors, particularly mosquitoes, through prevailing wind patterns, and it is the most important route of disease dissemination in countries sharing common borders.[42] The exchange of animals between various countries can also be a major cause of BEFV invasion in the reporting countries.[31]

The BEFV was reported from Surkhandarya region of Uzbekistan in August 2012, because of vector movements from neighboring countries like India, Japan and China, in which BEFV was already reported at different times.[3] In the current study, selected areas of Sindh, share border with neighboring countries like Afghanistan and India and have the same weather conditions. Therefore, these climatic patterns can generate a risk of BEFV introduction through insect vectors by crossing the shared borders between these countries. Moreover, the illegal transport of live animals from neighboring countries such as Iraq, Iran and Afghanistan on a large scale is also an important source for the spread of BEF infection in Pakistan.[28] The result of this study showed that the obtained isolates showed 99.00% homology with those from neighboring countries (Iran, India), suggesting that the same virus may be circulated through animal movement or vector transmission. Furthermore, Sindh province is situated in the subtropical region of Pakistan, having high humidity, an extensive canal-based irrigation system and post-monsoon stagnation provides a conducive environment for the growth of vector population, thereby playing a major role in the propagation of arboviral infections.

Practical measures should focus on vaccination of high-risk groups such as older animals and high producers, 1 - 2 month prior to the onset of the monsoon season. Vector management strategies are also essential during peak transmission season in a subtropical climate. Proper use of insecticides is highly effective in controlling the disease, along with efforts to eliminate breeding sites thoroughly, drainage of stagnant water, and maintenance of proper hygiene conditions. Utilizing local breeds such as Kundi and Red Sindhi, which are better adapted to the local environment, can also help reduce disease susceptibility. For crossbred animals, provision of shaded areas and cooling system is crucial to minimize stress and mitigate the infection. Most importantly, educating local farmers about disease detection and reporting is very critical to enhance control and prevention efforts. Collectively, these strategies can contribute to effective control in subtropical climate.

Bovine ephemeral fever is a re-emerging disease-causing significant economic loss among cattle and buffalo worldwide. We identified the BEFV from Sindh, Pakistan, marking a starting point for understanding the epidemiology of BEF in this region, filling a long-lasting gap in national surveillance. The phylogenetic analysis revealed that Pakistani isolates resemble strains from Iran, India, and Australia. The overall prevalence of BEF was 13.83% in the studied areas, with a higher prevalence in cattle (15.49%) compared to buffaloes (3.70%). Risk analysis indicated that high-producing animals in peak lactation and those reared on grazing were more susceptible to infection, especially after heavy monsoon rains in September. The obtained sequences are a preliminary insight, and future studies at the national level should include other provinces to obtain larger virus samples (kb) to obtain larger viral genome segments for whole-genome sequencing of BEF in Pakistan. This would enable a more detailed understanding of the virus, facilitating the development of effective diagnostic and control measures.

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Conflict of interest

The authors declare no conflict of interest.

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